

Efficient removal of PCR inhibitors using agarose-embedded DNA preparations

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ABSTRACT

The use of agarose blocks containing embedded DNA improves the PCR amplification from templates naturally contaminated with polysaccharides or humic acids, two powerful PCR inhibitors. Presumably, the difference in size between the DNA macromolecules and these contaminants allows their effective removal from the agarose blocks by diffusion during the washing steps, whereas genomic DNA remains trapped within them. In addition, agarose-embedded DNA can be directly used for PCR since low melting point agarose does not interfere with the reaction. This simple and inexpensive method is also convenient for genomic DNAs extracted by other procedures, and it is potentially useful for samples containing other kinds of soluble inhibitors, overcoming this important problem of current amplification techniques.

Organic and inorganic compounds that inhibit the amplification of nucleic acids by PCR are common contaminants in DNA samples from various origins. They can interfere with the reaction at several levels, leading to different degrees of attenuation and even to complete inhibition. This constitutes an important problem for general research, and especially for clinical, forensic and environmental screening. A wide variety of PCR inhibitors have been reported, and they appear to be particularly abundant in complex samples such as animal fluids, food, organic soils or samples containing high bacterial concentrations (reviewed in 1). Most of them (e.g. polysaccharides, urea, humic acids or hemoglobin) exhibit similar solubility to DNA. As a consequence, they are not completely removed during classical extraction protocols (such as detergent, protease and phenol-chloroform treatments), remaining as contaminants in the final DNA preparations. Several methods have been developed to avoid these contaminations. Some of them, such as dialysis against large volumes of solvents or centrifugation in CsCl gradients (2), are simple but imply the loss of non-negligible amounts of the original sample and other materials. Others are very specific methods directed only against concrete contaminants and usually require expensive materials. Several examples are ion-exchange columns, glass bead extraction, immunomagnetic separation, size-exclusion chromatography, anion-binding resins or spin columns (1). Furthermore, these additional manipulations increase cross-contamination risks and the subsequent false-positive results (3).

As an alternative to eliminate the presence of inhibitors, a simple DNA extraction method, originally developed to recover intact

DNA macromolecules (e.g. complete prokaryotic or eukaryotic chromosomes) for pulsed-field gel electrophoresis analysis, has been tested in this work. Basically, whole cells are embedded in low melting point (LMP) agarose blocks and then immersed in a lysis buffer. By this procedure, intact genomic DNA (gDNA) can be obtained avoiding shear damage (4). Several washing steps can be carried out prior to other treatments (such as endonuclease digestion). Taking advantage of the important difference of size between DNA macromolecules and most common soluble PCR inhibitors, I have adapted this method to the preparation of high quality gDNA for PCR amplification. Large DNAs remain trapped within the agarose blocks, whereas cell debris and contaminants are free to diffuse during lysis and washing steps. In this way, the resulting gDNA is highly purified and free of contaminants. In addition, agarose-embedded DNA is useful for PCR, since reactions are not affected by the presence of high quality LMP agarose concentrations even as high as 0.3% in the PCR mixture (data not shown).

This method was tested using gDNA naturally contaminated with polysaccharides and humic acids, which are especially fastidious contaminants and difficult to remove using conventional protocols. gDNA was prepared from the bacterium *Enterobacter aerogenes*, protoplasts of the brown alga *Pylaiella littoralis* (kindly provided by Dr G.Ducieux) and unidentified bacteria naturally present in a humus-rich forest soil sample. Cell walls of *E.aerogenes* (5) and *P.littoralis* (6) are rich in several polysaccharides, whereas humic acids are very abundant in organic soils (1). *Enterobacter aerogenes* was grown in LB medium supplemented with 20 mM sodium citrate and cells were collected by direct centrifugation at 10 000 g for 10 min. *Pylaiella littoralis* cells were collected by centrifugation at 2000 g for 5 min. Soil samples were suspended in 5 vol of 0.125 M EDTA pH 8.0, maintained overnight in an orbital shaker at 150 r.p.m. to detach bacterial cells from sand and organic debris, and filtered through a nylon gauze to eliminate big particles. A low-speed centrifugation step at 1500 g for 10 min was carried out for further removal of debris. The supernatant was then centrifuged at 10 000 g for 10 min to collect bacterial cells.

Cells were included in agarose plugs and gDNA prepared following the method of Schwartz and Cantor (4) with several modifications. Cell pellets were resuspended in 10 mM Tris-HCl pH 8.0, 1 M NaCl to achieve a cell density of $\sim 2 \times 10^5$ cells/ml. One volume of melted 1.6% LMP agarose was then added and gently mixed. The mixture was poured into sterile 100 μ l rectangular moulds prior to solidification. Agarose blocks were extracted from moulds and the embedded cells were lysed by overnight incubation at 50 °C in a 0.01 M Tris, 0.5 M EDTA pH 9.2,

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1% Lauroyl sarcosine and 2 mg/ml proteinase K solution (~1 ml of solution per agarose block). Subsequently, lysis buffer was removed and replaced by excess volume of TE buffer (~2 ml per block). After two extensive washes in TE (5 h with gentle shaking), highly purified gDNA remained trapped in the agarose matrix.

Cell aliquots of the assayed organisms were subjected in parallel to four different conventional DNA purification protocols to perform a comparative analysis. These were: (i) classical lysis with SDS and proteinase K followed by phenol–chloroform purification (7), and additional (ii) purification using the GeneClean procedure (BIO 101 Inc., Vista, CA); (iii) the method of Saghai-Marooof *et al.* (8) using hexadecyltrimethylammonium bromide (CTAB); and (iv) the Wizard Genomic DNA Purification System kit (including a high-salt purification step) as recommended by the manufacturer (Promega, Madison, WI). DNA concentration in these aqueous solutions was measured spectrophotometrically and adjusted to 50 ng/μl with TE.

PCR assays were carried out with the obtained gDNAs as templates in 50 μl test volumes using 2.5 U *Taq* DNA polymerase (Promega, Madison, WI), 1.5 mM MgCl₂, 0.2 mM of each dNTP and 0.4 μM of each primer. For gDNAs in aqueous solution, 50 ng were used by reaction, whereas for agarose-embedded gDNA, 5 μl fragments (cut with a sterile surgical blade) were directly added to each PCR mixture. Reactions were designed to amplify a 0.6 kb fragment of the 16S rRNA gene from the bacterial gDNAs using the primers 926 SSU and 1492 SSU (9), and a 0.85 kb fragment of the elongation factor 1α (EF-1α) gene from the *P.littoralis* gDNA using the primers 1F and 7R (10). The thermocycling reaction parameters were always 35 cycles, each cycle consisting of 1 min at 95°C for denaturation, 1 min at 50°C for annealing and 1 min at 72°C for extension. The products were electrophoresed on 1.2% agarose gels.

Figure 1A shows the results of these amplifications. All PCRs from gDNAs extracted following conventional methods failed to amplify the expected fragments. It is very likely that they were completely inhibited by the presence of contaminating remnants of polysaccharides or humic acids. Only the PCRs from agarose-embedded gDNA showed the desired amplification bands. The identity of these bands was corroborated by Southern blotting and hybridisation with 16S rRNA or EF-1α specific probes (data not shown).

A further application of this method consisted of the preparation of agarose-embedded gDNA from the DNA aqueous solutions used in the previous experiments. This was done by addition of 1 vol of 1.6% melted LMP agarose to each gDNA aliquot; these were then poured into moulds until solidification. The obtained blocks were washed in TE as described above. Large-size gDNA fragments were thus retained within the agarose matrix, whereas small-size fragments, as well as the soluble contaminants, diffused outside plugs. PCRs were subsequently carried out using 2.5 μl gDNA-containing agarose slices as templates. Positive results were obtained in all amplifications (Fig. 1B), which demonstrates that PCR inhibitors present in the original gDNA samples had been removed. Interestingly, control samples obtained from cells directly embedded in agarose showed the best results again (lane 5), probably because samples prepared from gDNA aqueous solutions have lost small fragments by diffusion outside blocks and thus they contained a smaller proportion of intact gDNA.

In summary, extensively washed agarose-embedded gDNA provides clean, high quality template gDNA for PCR purposes. This method can be used as an effective and reproducible

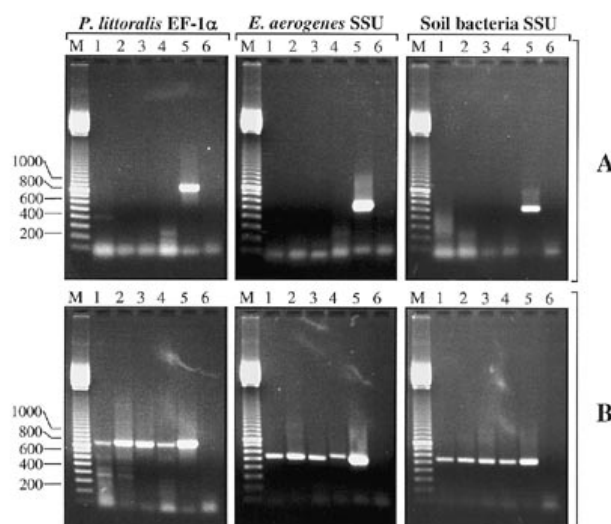


Figure 1. Effect of polysaccharide (*P.littoralis* and *E.aerogenes*) or humic acids (soil bacteria) contamination on PCR amplifications from different genomic DNA extractions. (A) Amplifications from DNAs extracted by a conventional method (lane 1), GeneClean (lane 2), CTAB treatment (lane 3), Wizard Genomic DNA Purification System (lane 4) and LMP agarose procedure (lane 5). Lane 6 shows a negative control without DNA. (B) Amplifications from the same DNAs after their inclusion in LMP agarose blocks and extensive washing with TE. The size marker is a 100 bp ladder (lane M); sizes are indicated in bp.

alternative to classical improved purification protocols, with the additional advantage of avoiding considerable DNA losses and the use of large dialysis volumes or very specific and usually expensive materials. It requires a small number of steps and manipulations, thus diminishing the risk of contamination by foreign DNA. It can be successfully applied to gDNA already extracted by other methods, making it convenient for the purification of gDNA from sources inadequate for direct agarose embedding (e.g. animal or plant tissues). In addition, it may be potentially useful for the removal of other soluble PCR inhibitors present as natural contaminants of DNA samples.

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